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4. Title of the invention	Recombinant Protein Expression
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5. Name of your agent <i>(if you have one)</i>	Carpmaels & Ransford
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Recombinant Protein Expression

The invention relates to methods for increasing the yield of folded recombinant protein in host cells.

All publications, patents and patent applications cited herein are incorporated in full by
5 reference.

The overproduction of recombinant proteins in cellular systems frequently results in misfolding of these proteins. The fates of the misfolded recombinant proteins differ. They may refold to the native state or be degraded by the proteolytic machinery of the cell or be deposited into biologically inactive large aggregates known as 'inclusion bodies'.

- 10 The folding of proteins and the refolding of misfolded soluble and aggregated proteins is known to be mediated by a network of evolutionarily conserved protein molecules called chaperones (Hartl, F.U., *Nature*, 381, 571-580, (1996); Horwich, A.L., Brooks Low K., Fenton, W.A., Hirshfield, I.N. & Furtak, K., *Cell* 74, 909-917 (1993); Ellis, R.J. & Hemmingsen, S.M., *TiBS*, 14, 339-342, (1989); Bukau, B., Hesterkamp, T. & Lührink, J.,
15 *Trends Cell Biol.*, 6, 480-486, (1996); Bukau, B., Deuerling, E., Pfund, C. & Craig, E.A., *Cell*, 101, 119-122, (2000)). Major chaperones include members of evolutionarily conserved protein families, including the Hsp60 family (which includes the bacterial chaperone GroEL), the Hsp70 family (which includes the bacterial chaperone DnaK), the Hsp100 family (which includes the bacterial chaperone ClpB), the Hsp90 family (which
20 includes the bacterial chaperone HtpG), the bacterial Trigger factor family, and the small HSPs (which includes the bacterial proteins IbpA and IbpB).

- Bacterial systems like the gram-negative bacterium *Escherichia coli* are a popular choice for the production of recombinant proteins. In *E. coli*, it is known that the DnaK and GroEL/ES chaperone systems assist the *de novo* folding of proteins (Hartl, F.U., *Nature*,
25 381, 571-580, (1996); Ewalt, K.L., Hendrick, J.P., Houry, W.A. & Hartl, F.U. *Cell* 90, 491-500 (1997); Bukau, B., Deuerling, E., Pfund, C. & Craig, E.A., *Cell*, 101, 119-122, (2000); Teter, S.A. et al., *Cell*, 97, 755-765, (1999); Bukau, B. & Horwich, A.L., *Cell*, 92, 351-366, (1998); Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A. & Bukau, B. *Nature* 400, 693-696 (1999)).

- 30 Furthermore, DnaK and its co-chaperones DnaJ and GrpE are presently considered to form the most efficient chaperone system for preventing the aggregation of misfolded proteins (Mogk, A. et al., *EMBO J.*, 18, 6934-6949, (1999); Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P. & Bukau, B., *Mol. Microbiol.*, 40, 397-413, (2001); Gragerov, A. et al.;

Proc. Natl. Acad. Sci. U.S.A. 89, 10341-10344 (1992)). Increased levels of GroEL and its co-chaperone GroES have been shown to prevent the heat induced aggregation of proteins in cells deficient of other major chaperones (Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P. & Bukau, B., *Mol. Microbiol.*, 40, 397-413, (2001); Gragerov, A. et al.,
 5 *Proc. Natl. Acad. Sci. U.S.A.* 89, 10341-10344 (1992)).

Moreover, the disaggregation of protein aggregates in *E. coli* using chaperones has been proven for many different cellular proteins *in vivo* (Mogk, A. et al., *EMBO J.*, 18, 6934-6949, (1999)), as well as *in vitro* using thermolabile malate dehydrogenase (MDH) as a reporter enzyme (Goloubinoff, P., Mogk, A., Peres Ben Zvi, A., Tomoyasu, T. & Bukau,
 10 B., *Proc. Natl. Acad. Sci., USA* 96, 13732-13737, (1999)). Protein disaggregation is achieved by a bi-chaperone system, consisting of ClpB and the DnaK system. Large aggregates of MDH could be resolubilised *in vitro* and MDH was refolded afterwards into its native structure. Importantly, only the combination of both chaperones is active in resolubilisation and refolding of aggregated proteins. A recent publication showed that the
 15 resolubilisation of recombinant proteins from aggregates *in vivo* is possible. In these experiments, protein aggregates were generated by temperature upshift, and the solubilisation and refolding of these proteins was measured in the presence of protein synthesis inhibitors to ensure that only the pre-existing aggregated proteins were monitored. Molecular chaperones were able to resolve the aggregates under these
 20 conditions.

Previous studies also indicate that the solubility and yield of recombinant proteins could be enhanced by the overproduction of chaperones. Co-overproduction of GroEL/GroES enhanced the solubility of several recombinant proteins synthesised in *E. coli* (human ORP150, human lysozyme, p50^{est} protein tyrosine kinase, phosphomannose isomerase,
 25 artificial fusion protein PreS2-S'- β -galactosidase) (Amrein, K.E. et al., *Proc. Natl. Acad. Sci., USA* 92, 1048-1052 (1995); Nishihara, K., Kanemori, M., Yanagi, H. & Yura, T., *Appl. Environ. Microbiol.*, 66, 884-889 (2000); Thomas, J.G. & Baneyx, F., *Mol. Microbiol.* 21, 1185-1196 (1996); Proudfoot, A.E., Goffin, L., Payton, M.A., Wells, T.N. & Bernard, A.R. *Biochem J* 318, 437-442. (1996); Dale, G.E., Schönfeld, H.J., Langen, H.
 30 & Stieger, M., *Protein Engineering*, 7, 925-931 (1994)). The overproduction of the DnaK system together with recombinant target proteins elevates the solubility of endostatin, human ORP150, transglutaminase and the fusion protein PreS2-S'- β -galactosidase (Nishihara, K., Kanemori, M., Yanagi, H. & Yura, T., *Appl. Environ. Microbiol.*, 66, 884-889, (2000); Thomas, J.G. & Baneyx, F., *J Biol Chem* 271, 11141-11147 (1996);

Yokoyama, K., Kikuchi, Y. & Yasueda, H., *Biosci. Biotechnol. Biochem.* 62, 1205-1210 (1998)).

- So far, no systematic approach has been made, to analyse whether the combination of all three chaperones systems (DnaK, DnaJ GrpE; GroES, GroEL and ClpB) expressed together with target genes in *E. coli* cells enhances solubility of recombinant proteins. Furthermore, none of the above-described studies allows the widespread optimisation of expression systems that is required to improve yields of soluble proteins on a general level. For example, each of the prior investigations focused on only one or a very small number of target proteins. These investigations also focused on the use of only one or two combined chaperone systems. In addition, none of these investigations addressed the issue of the importance of the ratio of the chaperones to one another and to the recombinant target protein. The previous studies therefore did not provide any understanding of the relationship between different chaperone proteins with respect to the folding/refolding of recombinant target proteins.
- Accordingly, there remains a great need in the art for a general method to improve the yield of soluble recombinant protein in a given expression system. Such a method would allow the optimisation of expression systems to give maximal yields of soluble target proteins, and be of obvious industrial and commercial benefit.
- The present invention is based upon the systematic engineering of cells for the controlled co-overexpression of different combinations of chaperone genes and target genes. In addition, it was investigated whether disaggregation and refolding of recombinant proteins from aggregates/inclusion bodies could be stimulated by enhanced levels of chaperones when the production of the target protein is stopped. As a result, the invention provides novel methods of optimising a given expression system in order to achieve very high yields of the desired soluble recombinant protein.

According a first aspect of the present invention, there is provided a method for the expression of a recombinant protein of interest, said method comprising:

- a) culturing a host cell which expresses:
- i) one or more genes encoding one or more recombinant protein(s) of interest;
 - ii) at least two genes encoding proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and

their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression; and

b) separating said recombinant protein of interest from the host cell culture.

Through the recombinant engineering of host cells in this manner, the invention provides novel methods for producing a recombinant protein of interest, which have been found to lead to significant improvements in the levels of protein produced in the system. The mechanism is thought to be through increasing the folding rates of particular proteins using the co-expression of particular chaperones in controlled amounts. Using this system, very high yields of the desired soluble recombinant proteins of interest can be obtained.

Any recombinant protein of interest may be produced using the system of the invention. Preferred examples of proteins of interest will be apparent to the skilled reader. Particularly preferred recombinant proteins are those for which it is desirable to produce a large amount, and those of commercial interest.

Furthermore, the invention is readily applicable to a wide range of known expression systems by alterations in the cell culture techniques employed. For example, anaerobic fermenter-based cell culture would be appropriate for the culture of obligate anaerobes, whereas standard aerobic cell culture techniques would be appropriate for obligate aerobes. The nutrient composition of the culture medium may also be varied in accordance with the chosen expression system. The most suitable method of cell culture for a given expression system will be readily apparent to the skilled man.

Preferably, the genes selected in step a) ii) include DnaK, DnaJ and GrpE or homologs thereof, and may additionally include ClpB or a homolog thereof.

In another preferred aspect of the invention, the genes selected in step a) ii) include GroES and GroEL or homologs thereof.

More preferably, the genes selected in step a) ii) include the DnaK, DnaJ, GrpE, ClpB, GroES and GroEL genes or homologs thereof.

The above combinations of chaperone proteins have been found to be particularly suitable for use in the methods according to the invention.

For the purposes of this patent specification, two genes or proteins are said to be 'homologs' if one of the molecules has a high enough degree of sequence identity or similarity to the sequence of the other molecule to infer that the molecules have an equivalent function. 'Identity' indicates that at any particular position in the aligned sequences, the amino acid or nucleic acid residue is identical between the sequences.

'Similarity' indicates that, at any particular position in the aligned sequences, the amino acid residue or nucleic acid residue is of a similar type between the sequences. Degrees of identity and similarity can be readily calculated (Computational Molecular Biology, Lesk A.M., ed., Oxford University Press, New York, 1988; Biocomputing, Informatics and
 5 Genome Projects, Smith, D.W., ed., academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, New Jersey, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991).

- 10 The chaperone proteins for use in the invention therefore include natural biological variants (for example, allelic variants or geographical variations within the species from which the genes are derived) and mutants (such as mutants containing nucleic acid residue substitutions, insertions or deletions) of the genes. For the purposes of this application, greater than 40% identity between two polypeptides is considered to be an indication of
 15 functional equivalence. Preferred polypeptides have degrees of identity of greater than 70%, 80%, 90%, 95%, 98% or 99%, respectively. It is expected that any protein that functions effectively as a chaperone, or as part of a chaperone system, within the host cells of the expression system will be of value in the described methods.

- Preferably, the levels of the respective chaperone proteins are controlled in conjunction
 20 with the methods described above. Preferably, the levels of chaperone proteins are controlled by expressing the genes encoding the respective chaperone proteins from different promoters. Preferably, a selection or all of the promoters used are inducible. Different promoters may have different strengths and may respond to the same induction agent with different kinetics or be responsive to a different induction agent, allowing
 25 independent control of the expression level of each chaperone protein. Suitable promoters will be apparent to those of skill in the art and examples are given in standard textbooks, including Sambrook et al., 2001 (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); Ausubel et al., 1987-1995 (Current Protocols in Molecular Biology, Greene Publications and Wiley Interscience, New York,
 30 NY). Examples of suitable promoters include IPTG-regulated promoters, such as the PA11 and lac-O1 promoters (see Tomoyasu, 2001).

Alternatively, or in addition, the respective chaperone proteins are expressed using expression systems of different strength. Examples of different expression systems will be

clear to those of skill in the art; discussion of such systems may be found in standard textbooks, including Sambrook et al., 2001 (*supra*) and Ausubel et al., (*supra*). For example, the plasmid vector of the expression system may be a high copy number or low copy number plasmid. For instance, examples of *E.coli* compatible low copy number
5 plasmids include pSC101 and p15A ori.

Preferably, the chaperone proteins are over-expressed relative to the expression levels that occur naturally in non-recombinant cells.

Similarly, the invention provides for the levels of the chaperone proteins relative to the recombinant protein(s) of interest to be controlled by expressing the genes encoding the
10 respective proteins from different promoters, for the reasons described above. In addition, the expression of the chaperones and of the recombinant proteins(s) can be controlled using different polymerases.

In a second aspect, the invention also provides methods comprising the use of a block in protein synthesis during the culturing step a) described above. Preferably, the block in
15 protein synthesis is imposed by addition of an effective amount of a protein synthesis inhibitor to the culture system, once a desired level of recombinant protein of interest has accumulated. More preferably, the chosen protein synthesis inhibitor is chloramphenicol or tetracycline. In order to ensure that protein synthesis is adequately inhibited, an effective amount of a protein synthesis inhibitor should be added. Details of effective amounts of
20 protein synthesis inhibitor will be apparent to the skilled reader and are noted in standard textbooks. For example, for use in prokaryotic host cell systems, 200 μ g/mL chloramphenicol is effective to inhibit protein synthesis.

Any other method that inhibits protein synthesis may also be of value for use with the methods of the invention. This includes the use of mutant strains that are conditionally
25 defective in protein synthesis, for example because of the temperature sensitivity of an enzyme involved in plasmid or host cell DNA replication or in target gene and host gene transcription or in protein translation. The imposition of such a block in protein synthesis has been found to lead to significant increases in the level of recombinant protein that is generated in the system of the invention.

30 Alternatively, or in addition, the invention also provides for the use of a reduction in gene transcription, by removal of any agents that are effective to induce recombinant protein expression (such as IPTG for Lac repressor controlled genes), once a desired level of

recombinant protein of interest has accumulated. Alternatively, a reduction of construct transcription could be achieved via the addition of a transcription blocking compound (such as glucose for catabolite repressable genes).

This aspect of the invention thus provides a method for the expression of a recombinant protein of interest, said method comprising:

a) culturing a host cell which expresses:

i) one or more genes encoding one or more recombinant protein(s) of interest;

ii) one or more genes encoding one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression;

b) imposing a block in protein synthesis, for example by addition of an effective amount of a protein synthesis inhibitor to the culture system, once a desired level of recombinant protein of interest has accumulated; and

c) separating said recombinant protein of interest from the host cell culture.

Also provided is a method for the expression of a recombinant protein of interest, said method comprising:

a) culturing a host cell which expresses:

i) one or more genes encoding one or more recombinant protein(s) of interest;

ii) one or more genes encoding one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression;

b) imposing a reduction in gene transcription, for example by removal of any agents that are effective to induce recombinant protein expression (such as IPTG for Lac repressor controlled genes), or via the addition of a transcription blocking compound (such as glucose for catabolite repressable genes), once a desired level of recombinant protein of interest has accumulated; and

c) separating said recombinant protein of interest from the host cell culture.

Preferably, a combination of chaperone proteins is expressed as described above.

Preferably, the chosen protein synthesis inhibitor is chloramphenicol or tetracycline.

Preferably, in the methods of the above-described aspects of the invention the cultured host cell is a prokaryotic cell, such as an *E. coli* cell, a *Lactococcus* cell, a *Lactobacillus* cell or
5 a *Bacillus subtilis* cell, or a eukaryotic cell such as a yeast cell, for example a *Pichia* or *Saccharomyces* yeast cell, or an insect cell, for example after baculoviral infection.

Preferably, an optimised yield of recombinant protein of interest is manifested by increasing the level of *de novo* protein folding.

An optimised yield of said recombinant protein of interest may also be manifested by
10 increasing the level of *in vivo* refolding of aggregated, or misfolded soluble, recombinant protein.

An optimised yield of said recombinant protein of interest may also be manifested by increasing the level of *in vitro* refolding of aggregated, or misfolded soluble, recombinant protein.

15 An optimised yield of said recombinant protein may also be manifested by increasing the level of *de novo* protein folding in combination with increasing the increased level of *in vivo* refolding and/or *in vitro* protein refolding.

Preferably, said increased level of folding or refolding results in increased solubility of the recombinant protein of interest.

20 Preferably, said increased level of folding or refolding results in increased activity of the recombinant protein of interest.

According to a third aspect of the present invention there is also provided a method for increasing the degree of refolding of a recombinant protein of interest, said method comprising adding a composition containing a chaperone protein to a preparation of the
25 recombinant protein of interest *in vitro*. This has been found to increase significantly the degree of refolding of protein in preparations containing wholly or partially unfolded protein. The preparation of the recombinant protein of interest may be any preparation that contains protein that is partially or wholly unfolded or misfolded. Preferably, the preparation is a cell extract preparation, such as a lysate of a prokaryotic cell.

Preferably, a combination of chaperone proteins as described above is added to the preparation of the recombinant protein of interest.

The preparation of the recombinant protein of interest may be a preparation of soluble recombinant protein that has been precipitated *in vivo*, or may be a preparation of *in vitro* precipitated recombinant protein (for example, a host cell extract containing the

5 recombinant protein aggregate).

Preferably, said composition containing the chaperone protein(s) is added after removal of any agents that are effective to induce soluble recombinant protein expression (such as IPTG for Lac repressor controlled genes) or after addition of a transcription blocking

10 compound (such as glucose for catabolite repressable genes).

Preferably, the third aspect of the invention is used in conjunction with imposing a block in protein synthesis, for example by addition of an effective amount of a protein synthesis inhibitor to the culture system. As described above, chloramphenicol and tetracycline are examples of suitable protein synthesis inhibitors.

15 Preferably, when practising the above-described methods, the time course of refolding and the temperature at which refolding occurs is controlled. The time course of refolding and temperature at which it occurs are known to have a significant effect on the yield of soluble recombinant protein, and are thus an important aspect of a given expression system to be optimised for the maximal yield of soluble recombinant protein.

20 Various aspects and embodiments of the present invention will now be described in more detail by way of example. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

Brief description of the Figures

Figure 1A shows chaperone co-overproduction systems tested in *E. coli*. Genes encoding

25 three different chaperone-systems (GroEL/ES; DnaK, DnaJ, GrpE; and ClpB) were cloned in a pair of low copy number vectors, which are compatible with *E. coli* (pSC101 and p15A ori), carry the *lacI^Q* gene and different resistance markers for selection. Chaperone genes are set under the control of IPTG-regulated promoters (PA11/lacO1) for controlled expression. Each combination of vector pairs (1 to 5) differs in its combination and level of

30 chaperone expression. In these strains subsequently a third plasmid encoding a substrate protein was introduced.

Figure 1B shows chaperone expression patterns. The chaperone combinations 2 to 5 are shown. The left hand lane of each pair is loaded with a sample for which expression of the recombinant proteins had not been induced. The right hand column for each chaperone combination shows an IPTG-induced sample.

- 5 **Figure 2.** Chaperone and target protein co-expression under IPTG control. The target proteins Tep4, Btke and Lzip were purified by metal affinity chromatography after transformation in BL21(DE3) cells used as a control (K) and in the same strain but co-expressing the 5 different chaperone combinations reported in Figure 1.

- Figure 3.** *In vivo* induced refolding. **Figure 3A** shows the Btke expression level after
10 chaperone-induced re-folding in BL21(DE3) cells used as a control (K) and in the same strain but co-expressing the 5 different chaperone combinations reported in Figure 1. Cells were grown at 30°C, induced with 0.1 mM IPTG, grown overnight, and then either grown 2 more hours (first lane of each combination) or pelleted, re-suspended in fresh medium plus 200µg/mL chloramphenicol and cultured 2 more hours (second lane). **Figure 3B**
15 shows optimisation of the re-folding conditions using the chaperone combination 4 shown in Figure 3B. After overnight culture at 20°C the cells were pelleted, resuspended in fresh medium and cultured 1h, 2h, 3h, and 4h at 20°C (1 to 4), or 1h and 2h at 37°C (5 and 6) in the presence of 200µg/mL chloramphenicol. For each combination the first lane was loaded with the uninduced sample and the second with the treated one. **Figure 3C** shows
20 Btke expressed in control (C1) and chaperone combination 4 (C2) cells. Lanes were loaded with uninduced samples (K), induced and cultured at 20°C overnight plus two hours at the same temperature, pelleted after overnight growth, resuspended in fresh medium plus 200µg/mL chloramphenicol and cultured 2 more hours, as in 2 but in the presence of 1mM IPTG instead of chloramphenicol, resuspended in fresh medium for 1h, 2h, and 4h. The
25 numbers shown below the gel image indicate the increase factor obtained comparing the intensity of the bands to the reference (induced cells without chaperone co-expression). **Figure 3D** shows the effect of growth conditions on re-folding efficiency of Btke. Cells were grown overnight at 20°C (D1) and at 42°C before inducing the re-folding at 20°C (D2). Lanes were loaded with un-induced samples (K), induced and cultured overnight
30 plus two hours (1), resuspended in fresh medium plus 2h culture (2), in fresh medium plus 200 µg/mL chloramphenicol and cultured 2 more hours (3). **Figure 3E** shows the re-folding efficiency of Tep4 expressed in control (E1) and chaperone combination 4 (E2)

cells. Lanes were loaded with uninduced samples (K), induced and cultured overnight plus two hours (1), resuspended in fresh medium plus 2h culture (3), in fresh medium plus 200 μ g/mL chloramphenicol and cultured 2 more hours (4).

Fig. 4. *In vitro* re-folding. **Figure 4A** shows Btke expressed either in control cells (c) or in cells co-expressing chaperone combination 3 or 4. 3h after IPTG induction, cells were harvested and lysate prepared as described above. Samples containing 100 μ g lysate were supplemented with 10mM ATP and 3mM PEP and 20ng/ml PK. After indicated timepoints, soluble Btke protein was isolated and analysed by SDS-PAGE and Coomassie staining. **Figure 4B** shows the results produced when pellets with insoluble Btke were isolated from control cells. Pellets were suspended in buffer and where indicated chaperones were added. After 5 min, 2, 4, and 20 h soluble Btke protein was isolated as described above and analysed by SDS-PAGE and silver staining.

Examples

Examples 1-5 below illustrate the materials and methods used to investigate the effect of co-expressing different chaperone combinations on the yield of a large variety of different recombinant proteins.

Example 1: Construction of Chaperone Vectors

Plasmids carrying chaperone genes under the control of the IPTG-sensitive promoter PA1/lacO-1 were constructed as described (Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P., Buckau, B., Mol. Microbiol., 40, 397-413, (2001)). Target protein vectors were delivered to the Protein Expression Unit from different research groups working at the European Molecular Biology Laboratory.

Example 2: Transformation procedure

Competent BL21 (DE3) and Top10 cells were transformed with the following couples of plasmids for selective expression of chaperone combinations (Fig.1A). The clones for DnaK, DnaJ, and GrpE were carried by pBB530 and pBB535; the co-expression of DnaK, DnaJ, GrpE, and ClpB was regulated by pBB535 and pBB540; GroEL/ES system was expressed by pBB528 and pBB541; a large amount of the complete system DnaK, DnaJ, GrpE, ClpB, and GroEL/ES was ensured by pBB540 and pBB542; finally, a lower expression level of the same chaperone combination was obtained using pBB540 and pBB550. A complete array of single chaperone plasmid transformed cells was also

prepared as a control. Transformed cells were checked for chaperone expression and successively made competent. The protease deficient strain BB7333 (MC4100 $\Delta clpX$, $\Delta clpP$, Δlon) was used for transforming the Btkp protein. These strains were also made competent and used for a further transformation with the target proteins.

5 Example 3: Cell cultures

Single colonies from the transformed cells were used to inject 3 mL of LB medium. Liquid cultures were performed initially at 37°C, then transferred to 30°C and finally transferred to 20°C. Using different times of incubation at the higher temperatures it was possible to reach the OD₆₀₀ of 0.8 at the same time for all the different cell strains cultured together for comparative expression assays. Protein expression was performed overnight by inducing gene transcription using 0.1 mM IPTG. 1.5 mL of the overnight culture of both IPTG-induced (hereafter termed 'induced') and control bacteria was directly centrifuged in an Eppendorf tube and the pellet frozen and stored at -20°C. Alternatively, the pellet was re-suspended in 3 mL of fresh medium and divided into two aliquots of 1.5 mL, with or without the addition of 200 µg/mL chloramphenicol. After 2h culture at 20°C the cells were harvested as described before. Inclusion body overproduction was obtained by culturing the bacteria at 42°C overnight after induction. Large scale cultures were grown in 2L flasks using 5 mL of overnight LB pre-culture to inoculate 500 mL of Terrific Broth.

Example 4: Protein purification and evaluation

Frozen bacterial pellets were re-suspended in 350 µL of 20 mM Tris HCl, pH 8.0, 2mM PMSF, 0.05% Triton X-100, 1µg/mL DNAase and 1 mg/mL lysozyme and incubated on ice for 30 min, with periodic stirring. The suspension was sonicated in water for 5 minutes, an aliquot (of homogenate) was stored and the rest was pelleted in a minifuge. An aliquot of the supernatant was preserved and the rest was added to 15 µL of pre-washed magnetic beads (Qiagen) and incubated further 30 min under agitation before being removed. Beads were washed 30 min with 20 mM K-phosphate buffer, pH 7.8, 300 mM NaCl, 20 mM imidazole, 8% glycerol, 0.2% Triton X-100 and later with PBS buffer plus 0.05% Triton X-100. Finally they were boiled in 12 µL SDS sample buffer and the samples loaded for SDS PAGE analysis, using a Pharmacia minigel system. Proteins were detected after coloration with Simply Blue Safestain (Invitrogen) following the manufacturer's instructions and the gels were recorded using a Umax Astra 4000U scanner. Bands corresponding to the proteins were analysed using the public NIH Image 1.62f software. Alternatively, protein

was eluted from washed beads using 30µL PBS buffer plus 0.5M imidazole and its relative concentration measured following its adsorbance at 280nm. The proper folding was evaluated by circular dichroism using a J-710 spectropolarimeter (Jasco).

Example 5: In vitro experiments

- 5 Cells were grown in LB and after inducing the synthesis of either Btke or Btke together with GroEL/ES (combination 3) or together with GroEL/ES, DnaK, DnaJ, GrpE, ClpB (combination 4) for 3h with 1mM IPTG at 37°C, lysates were prepared as described above. For refolding of Btke from inclusion bodies using total lysate, 10mM ATP, 3mM phosphoenole pyruvate (PEP) and 20 ng/ml pyruvate kinase (PK) were added and
10 incubated at 20°C. After 5 min, 2, 4, and 20 h soluble material was separated from insoluble fractions by centrifugation (15 min, 4°C, 10.0000rpm) and the soluble fraction was used to isolate target protein as described above.

For resolubilisation of isolated Btke aggregates with exogenous chaperone addition, 100µg of total lysate (isolated from cells with overproduced Btke) was centrifuged for 15 min and

- 15 pellets were resuspended in 20 mM Tris/HCl, 100mM KCl and 20 mM MgCl. Chaperones were added as indicated and samples incubated at 20°C for 5 min, 2, 4, 20h. Soluble material was separated from inclusion bodies by centrifugation and isolated as described.

- 20 Examples 6 to 9 below illustrate the optimisation of chaperone co-expression combinations and other experimental variables in order to greatly increase the yield of a large number of diverse recombinant proteins.

Example 6: Investigation of the Effect of Chaperone Combinations on *de novo* Protein Folding

- Five different combinations of plasmids encoding chaperone systems (GroEL/ES; DnaK, DnaJ, GrpE and ClpB) in different combinations and amounts under the control of IPTG
25 regulated promoters were introduced into BL21 (DE3) cells as illustrated in Fig. 1A. The degree of chaperone expression was shown to be very high (Fig. 1B). These cells were subsequently transformed with plasmids expressing substrate proteins in an IPTG controlled manner (Fig. 1A). Therefore, co-expression of chaperones and target proteins was obtained by simultaneous induction of all the promoters with IPTG. Co-expression of
30 chaperones together with 50 different target genes was tested. For each target protein, all five different chaperone combinations were tested and solubility of the recombinant proteins analysed. In summary a higher yield of soluble substrate protein was achieved in more than 50% of the tested constructs (see Table 1 below).

Table 1 shows a list of the proteins used in the survey for analysing the effect of chaperone co-expression on soluble target protein yield. The table shows the molecular weight of the constructs, the original organisms from which they were cloned, whether they corresponded to full length proteins (Fl) or to domains, expressed alone or fused to a partner (fus), and their cell localisation (cytoplasm, membrane, nucleus, secreted) *in vivo*. The yield increase factor (IF) induced by the best chaperone combination is reported under 'Chap. IF' and the yield increase factor obtained using the refolding protocol under 'Refolding IF'. The symbol (/) signifies that the experiment has not yet been done and (!) that protein has been obtained using constructs that gave no soluble protein when expressed in wild type bacteria.

Table 1:

Protein	MW	Organism	Features	Chap. IF	Refolding IF
GTR1	40 kD	S. cerevisiae	Fl/cyt	3	3
<u>BtKp</u>	55 kD	H. sapiens	domain/cyt	0	28
Xpot1	110 kD	H. sapiens	Fl/cyt	0	/
<u>XkdpA1</u>	62 kD	X. laevis	domain/fus/cyt	0	!
<u>XkdpB1</u>	40 kD	X. laevis	domain/fus/cyt	0	!
<u>HbpH</u>	9 kD	H. sapiens	domain/cyt	3.5	3.5
<u>TEVprotease</u>	30 kD	TEV	domain	3.5	/
<u>Pex5p</u>	50 kD	H. sapiens	domain/cyt	0	/
<u>UCP1</u>	33 kD	R. norvegicus	domain/membr	0	0
Transcr Fact	37 kD	H. sapiens	Fl/cyt	0	0
<u>BtKe</u>	55 kD	H. sapiens	domain/cyt	4	42
<u>XkdpA2</u>	38 kD	X. laevis	domain/fus/cyt	0	/
<u>XkdpB2</u>	35 kD	X. laevis	domain/fus/cyt	0	/
<u>XkdpA3</u>	72 kD	X. laevis	domain/fus/cyt	0	/
Rolled	43 kD	D. melanogaster	Fl/cyt	4.5	4.5
Lzip	41 kD	H. sapiens	Fl/cyt	!	/
1Ap	52 kD	D. melanogaster	Fl/nucl	0	/
Chip	64 kD	D. melanogaster	Fl/nucl	0	/

dLMO	37 kD	D. melanogaster	Fl/nucl	0	/
Tlc	57 kD	R. prowazekii	Fl/membr	0	/
BtKc	64 kD	H. sapiens	Fl/cyt	3	/
PhosphK	29 kD	H. sapiens	Fl/cyt	3	7
<u>Compl.Tep3</u>	47 kD	A. gambiae	domain/fus	4	/
<u>Compl.Tep4</u>	45 kD	A. gambiae	domain/fus	3.5	/
<u>XklpA4</u>	72 kD	X. laevis	domain/fus/cyt	2.5	2.5
<u>XklpB3</u>	73 kD	X. laevis	domain/fus/cyt	2.5	2.5
<u>E8R1</u>	58 kD	Vaccinia virus	Fl/membr/fus	7	/
<u>Compl.Tep3</u>	70 kD	A. gambiae	domain/fus/secret	0	11
<u>Compl.Tep4</u>	68 kD	A. gambiae	domain/fus/secret	3.5	13
<u>MaxF</u>	7.5 kD	synthetic	domain	3	/
<u>XklpA5</u>	35 kD	X. laevis	domain/fus/cyt	0	19
<u>E8R2</u>	85 kD	Vaccinia virus	Fl/membr/fus	5.5	5.5
<u>Susy</u>	90 kD	Z. mays	Fl/membrane	3	5
Mash	91 kD	Z. mays	Fl/cyt	0	3
PPAT	22 kD	E. coli	Fl/cyt	0	3
2Ap	54 kD	D. melanogaster	Fl/nucl	!	3
F10L	45 kD	Vaccinia virus	Fl/fus	0	0
B1R	47 kD	Vaccinia virus	Fl/fus	3.5	3.5
<u>1Frenge</u>	43 kD	D. melanogaster	domain/cyt	!	!
<u>Tep1</u>	7 kD	A. gambiae	domain/secret	3	6
<u>Tep2</u>	11 kD	A. gambiae	domain/secret	0	0
<u>2Frenge</u>	55 kD	D. melanogaster	domain/fus	0	2
GFP-fusion	95 kD	A. victoria	Fl/fus/cyt	0	0
2C18	50kD	H. sapiens	Fl/fus	3	8
22j21	72kD	H. sapiens	Fl/fus	!	!
<u>XklpA+B</u>	15+17kD	X. laevis	domain/complex	2.5	3.5
<u>Msl3</u>	14 kD	D. melanogaster	domain/cyt	2.5	2.5

<u>Mash+Susy</u>	94+90 kD	Z. mays	Fl/complex	3	3
<u>Endostatin</u>	22 kD	M. musculus	domain/secr	0	0
<u>Kringle</u>	30 kD	H. sapiens	domain/fus	0	0

As can be seen from the 'Chap. IF' ratings, soluble target protein yield increased between 2.5 and 7-fold. Effects of co-expressed chaperones were not limited to a certain type of substrate protein. The target proteins tested were representative of several different classes, including complexes, soluble, membrane-bound and secreted proteins, full-length, domains and fusion constructs, with a molecular weight spanning from 7.5 to 110 kD, expressed in the cytoplasm and in the periplasm (Table 1). Moreover, in some cases, like Lzip (see Table 1 and also Figure 2), co-expression of chaperones was the only possibility to obtain any soluble protein. Evaluation of the 23 positive cases indicated that the most efficient chaperone combination was the fourth, which expressed all three chaperone systems in large amounts, followed by the third, fifth, first and the second. Nevertheless, as is demonstrated in the case of LZip transcription factor where chaperone combination 1 worked far better than the others, any one chaperone combination is not necessarily optimal for all target proteins. Thus, despite the systematic approach it was not possible to infer general rules about the optimal conditions to succeed. No protein class showed better results in combination with particular chaperone combinations and no expression vector ensured significantly better yields. The only exception was when target proteins were cloned in high copy number vectors. In such a case no positive result was observed. The competition for the protein synthesis machinery could be considered as a reason, since chaperone expression is inhibited when a target protein was co-expressed and is completely prevented in cells harbouring expression vectors with pUC origin (data not shown). The results shown in Table 1 clearly demonstrate the very large increases in yield possible via the use of the disclosed methods.

Example 7: Testing the Effect of Co-overexpression of Chaperone Combinations and Target Proteins on Re-folding of Aggregated Proteins Using Chloramphenicol

In the experiments of Example 5 it was often observed that inclusion bodies accumulated even in the presence of overproduced chaperones increasing the amount of soluble proteins. A recent paper (Carrio, M. M. and Villaverde, A. FEBS Lett., 489, 29-33 (2001))

showed that soluble proteins could be recovered *in vivo* from inclusion bodies when the protein synthesis was blocked by chloramphenicol addition and the whole cellular folding machinery became available for precipitated proteins. Therefore, we investigated the overexpression of chaperones not only for keeping recombinant proteins soluble but also for increasing the re-folding capability of cells. To investigate this further, we co-overexpressed chaperones and target genes as described before. Subsequently, we stopped protein synthesis by the addition of chloramphenicol. Cells were transferred to fresh media, incubated at 20°C and resolubilisation of targets had been analysed at different time points. In fact, in the case of Btke the chloramphenicol-induced block of protein synthesis induced a low increment of the soluble recombinant protein in control cells but an impressive increase when specific chaperone combinations were co-expressed simultaneously with the target gene prior to the translational arrest (Fig. 3A). It is worthy to note that for Btke the optimal chaperone combination differed when the soluble protein accumulated during standard culture conditions and when protein synthesis has been blocked (Figs. 2 and 3A). The choice of time and temperature conditions during re-folding was crucial for optimising the result (Fig. 3B). Longer incubation times or higher temperature lowered the amount of recovered soluble protein, probably because degradation by proteases takes over re-folding activity. As can be seen in Table 1 above, this method of combining chaperone co-overexpression with the blocking of protein synthesis resulted in a great improvement in the yield of recombinant protein in a large number of the combinations tested.

Example 8: Testing the Effect of Co-overexpression of Chaperone Combinations and Target Proteins on Re-folding of Aggregated Proteins by Reducing Construct Gene Transcription

The protocol used to block protein synthesis, as described in Example 6 above, was evaluated by means of experiment. It was found that the original protocol can be simplified and that it was not strictly necessary to completely prevent protein synthesis in order to induce re-folding, and in fact the cessation of recombinant protein expression by removing the induction agent (IPTG) was sufficient. In this case the target protein could be re-folded to a level comparable to that obtained in the presence of chloramphenicol but only in the presence of the recombinant over-expressed chaperones (Fig. 3C). For Btke the optimal re-folding conditions enabled the recovery of 42-fold more protein than in the standard growth conditions using normal BL21 (DE3) cells and the simplified protocol (without chloramphenicol) gave an increase factor of 26. We also tried to induce the inclusion body formation culturing the bacteria at 42°C and starting the re-folding from a higher amount

of material but the improvement was negligible (Fig. 3D), probably indicating that the limiting factor is represented from the folding machinery or from the cellular degrading metabolism. These two factors seem to be somehow connected, as illustrated in the case of Tep4. In contrast to Btke this protein was expressed in soluble form at sufficient levels also
 5 at standard culture conditions and chaperone co-expression induced a limited yield increase (Fig. 3E). Nevertheless, the suppression of IPTG induction by simple exchange with fresh medium boosted the accumulation of soluble protein in both the strains but only the co-expression of recombinant chaperones could ensure the same results when chloramphenicol was added. Generally, we observed that the addition of fresh medium
 10 alone was more effective than the combination of fresh medium and chloramphenicol in strains with wild type chaperone expression. This indicates that the removal of the inducer IPTG, and the subsequent cessation of transcription of the target gene, is sufficient to allow refolding from inclusion bodies. It was a goal of the inventors to obtain more information about the relationship between protein re-folding and degradation by transforming our
 15 vectors in the protease deficient strain BB7333. However, the inventors were not able to raise a sufficient number of bacterial colonies. This finding confirmed the general role of proteases in maintaining cell viability (Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P., Buckau, B., Mol. Microbiol., 40, 397-413, (2001)) and suggests that a certain degree of protein degradation must be maintained. It is therefore clear from the above example that a
 20 reduction in recombinant target gene transcription can also allow the refolding of aggregated proteins to proceed, leading to greatly improved yields of the soluble recombinant protein of interest.

Example 9: The Effect of Co-overexpression of Chaperone Combinations and Target Proteins on Re-folding of Aggregated Proteins *in vitro*

25 Next, we analysed whether co-expressed chaperones are capable of enhancing the refolding of target proteins from inclusion bodies *in vitro* after cell lysis. For that purpose, we induced simultaneously synthesis of Btke together with either chaperone combination 3 or 4. Cells were harvested after induction and total lysates containing inclusion bodies and chaperones were isolated. Subsequently an ATP-regenerating system was added to the
 30 lysates and the soluble protein was purified after 5 min, 2h, 4h and 20h. Lysate containing the chaperone mixture 4, which was the most efficient during the *in vivo* refolding of Btke, showed already 5 min after the addition of ATP that approximately all Btke could be recovered in the soluble fraction. The control lysate, where only Btke was overexpressed, and the lysate with enhanced levels of GroEL/EL showed no significant recovery of

soluble Btke (Fig. 4A). It is therefore clear that co-overexpressed chaperone mixtures stimulate re-solubilisation of inclusion bodies from bacterial cell lysates. Refolding of Btke inclusion bodies was also possible when chaperones were added exogenously to isolated aggregates (Figure 4B). However, refolding efficiency was much lower and refolding kinetics much slower, most probably due to the limited amount of added chaperones. This example clearly shows that co-expression of chaperones can also increase the yield of soluble recombinant protein via an enhancement of the refolding of target proteins from aggregates/inclusion bodies *in vitro*.

The above Examples 1-8 have clearly shown the value of the methods provided by the present invention for increasing protein yield. The re-folding protocol applied to the chaperone transformed cells allowed even higher yields of soluble protein than the simple co-expression with the target proteins in 8 on 17 cases and, importantly, also gave positive results also in the case of 8 constructs insensitive to simple co-expression. Taking all the results together chaperones had a positive effect on soluble protein accumulation in 68% of the cases analysed in our survey. The ratio remains basically the same if all the 50 constructs are considered (34 positive) or if only the 37 different proteins are taken in account (24 positive, 65%). It must be remarked that such a positive result has been obtained despite the fact that most of the constructs used in the experiment correspond to sequences difficult to be expressed in a soluble form in bacteria, like membrane-associated or secreted proteins, regions not corresponding to structural domains or complexes (underlined in Table 1). The advantage of the *in vivo* disaggregation is that protein refolding follows native patterns and, therefore, recovers its native conformation. The correct folding of some of the proteins was analysed by purification until homogeneity followed by circular dichroism analysis, indicating that the proteins had adopted their native conformation after refolding. Importantly, the enzymatic activities of the kinases B1R and F10L, the TEV protease and luciferase were also recovered after re-folding (data not shown). Larger scale cultures confirmed the trend observed in test cultures, suggesting that the disclosed methods are suitable for industrial applications. In summary, the invention provides not only a method for the production of large amounts of soluble recombinant protein, but also a method for the production of large amounts of recombinant protein that is correctly folded and furthermore retains the native protein's biological activity.

CLAIMS

1. A method for the expression of a recombinant protein of interest, said method comprising:
 - a) culturing a host cell which expresses:
 - 5 i) one or more genes encoding the recombinant protein(s) of interest;
 - ii) at least two genes encoding proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein
10 expression; and
 - b) separating said recombinant protein of interest from the host cell culture.
2. A method according to claim 1, wherein the genes selected in step a) ii) include DnaK, DnaJ and GrpE or homologs thereof.
- 15 3. A method according to claim 2, wherein the genes selected in step a) ii) additionally include ClpB or a homolog thereof.
4. A method according to any one of claims 1-3, wherein the genes selected in step a) ii) include GroES and GroEL or homologs thereof.
5. A method according to claim 4, wherein the genes selected in step a) ii) include the
20 DnaK, DnaJ, GrpE, ClpB, GroES and GroEL genes or homologs thereof.
6. A method according to any one of the preceding claims wherein the levels of the respective chaperone proteins are controlled.
7. A method according to claim 6, wherein said levels of chaperone proteins are controlled by expressing the genes encoding the respective chaperone proteins from
25 different promoters.
8. A method according to claim 6 or claim 7, wherein the respective chaperone proteins are expressed using expression systems of different strength.
9. A method according to any one of claims 6-8, wherein said chaperone proteins are over-expressed relative to the expression levels that occur naturally in non-recombinant cells.
30

10. A method according to any one of the preceding claims, wherein the levels of the chaperone proteins relative to the recombinant protein(s) of interest are controlled by expressing the genes encoding the respective proteins from different promoters or by using different polymerases.

5 11. A method according to any one of the preceding claims, wherein in culturing step a) of the method, a block in protein synthesis is imposed, for example, by the addition of an effective amount of a protein synthesis inhibitor to the culture system, once a desired level of recombinant protein of interest has accumulated.

12. A method according to claim 11, wherein the chosen protein synthesis inhibitor is
10 chloramphenicol or tetracycline.

13. A method according to any one of claims 1-12, wherein in culturing step a) of the method, a reduction in gene transcription is imposed, for example, by removal of
any agents that are effective to induce recombinant protein expression (such as
IPTG for Lac repressor controlled genes), or via the addition of a transcription
15 blocking compound (such as glucose for catabolite repressable genes), once a
desired level of recombinant protein of interest has accumulated

14. A method for the expression of a recombinant protein of interest, said method comprising:

a) culturing a host cell which expresses:

20 i) one or more genes encoding the recombinant protein(s) of interest;

ii) one or more genes encoding one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for
25 example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression;

b) imposing a block in protein synthesis, for example, by the addition of an effective amount of a protein synthesis inhibitor to the culture system, once a desired level of recombinant protein of interest has accumulated; and

30 c) separating said recombinant protein of interest from the host cell culture.

15. A method for the expression of a recombinant protein of interest, said method comprising:

a) culturing a host cell which expresses:

- i) one or more genes encoding the recombinant protein(s) of interest;
- ii) one or more genes encoding one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression;

b) imposing a reduction in gene transcription, for example, by removal of any agents that are effective to induce recombinant protein expression (such as IPTG for Lac repressor controlled genes), or via the addition of a transcription blocking compound (such as glucose for catabolite repressable genes), once a desired level of recombinant protein of interest has accumulated; and

c) separating said recombinant protein of interest from the host cell culture.

16. A method according to claim 14 or claim 15, wherein in step a) ii), a combination of chaperone proteins is expressed as recited in any one of claims 2-5.

17. A method according to claim 14 or claim 16, wherein the chosen protein synthesis inhibitor is chloramphenicol or tetracycline.

18. A method according to any one of the preceding claims, wherein said cultured host cell is a prokaryotic cell, such as an *E. coli* cell, a *Lactococcus* cell, a *Lactobacillus* cell or a *Bacillus subtilis* cell, or a eukaryotic cell such as a yeast cell, for example a *Pichia* or *Saccharomyces* yeast cell, or an insect cell, for example after baculoviral infection.

19. A method according to any one of the preceding claims, wherein an optimised yield of said recombinant protein of interest is manifested by increasing the level of *de novo* protein folding.

20. A method according to any one of claims 1-19, wherein an optimised yield of said recombinant protein of interest is manifested by increasing the level of *in vivo* refolding of aggregated, or misfolded soluble, recombinant protein.

21. A method according to any one of claims 1-19, wherein an optimised yield of said recombinant protein of interest is manifested by increasing the level of *in vitro* refolding of aggregated, or misfolded soluble, recombinant protein.
22. A method according to claim 19, wherein an optimised yield of said recombinant protein is manifested by increasing the level of *de novo* protein folding in combination with an increased level of *in vivo* protein refolding and/or *in vitro* protein refolding.
23. A method according to any one of claims 19-22, wherein said increased level of folding or re-folding results in increased solubility of the recombinant protein of interest.
24. A method according to any one of claims 19-22, wherein said increased level of folding or re-folding results in increased activity of the recombinant protein of interest.
25. A method for increasing the degree of refolding of a recombinant protein of interest; said method comprising adding a composition containing a chaperone protein to a preparation of the recombinant protein of interest *in vitro*.
26. A method according to claim 25, wherein a combination of chaperone proteins as recited in any one of claims 2-5 is added to the preparation of the recombinant protein of interest.
27. A method according to claim 25 or claim 26, wherein the preparation of the recombinant protein of interest is a preparation of soluble recombinant protein that has been precipitated *in vivo*.
28. A method according to claim 25 or claim 26, wherein the preparation of the soluble recombinant protein of interest is a preparation of *in vitro* precipitated recombinant protein.
29. A method according to any one of claims 25-28, wherein said composition containing the chaperone protein(s) is added after removal of any agents that are effective to induce soluble recombinant protein expression (such as IPTG for Lac repressor controlled genes) or after addition of a transcription blocking compound (such as glucose for catabolite repressable genes).

30. A method according to any one of claims 25-29, additionally comprising the step of imposing a block in protein synthesis, such as by the addition of an effective amount of a protein synthesis inhibitor to the culture system.
31. A method according to claim 30, wherein the chosen protein synthesis inhibitor is
5 chloramphenicol or tetracycline.
32. A method according to any one of the preceding claims, wherein the refolding temperature and time course of refolding are controlled.

Abstract**Recombinant Protein Expression**

There are provided methods for the expression of a recombinant protein of interest, said methods comprising, in addition to various additional steps:

- 5 a) culturing a host cell which expresses:
- i) one or more genes encoding the recombinant protein(s) of interest;
 - 10 ii) at least two genes encoding proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression; and
- b) separating said recombinant protein of interest from the host cell culture.

Also provided are methods for increasing the degree of refolding of a recombinant protein of interest by adding a composition containing a chaperone protein to a preparation of the recombinant protein of interest *in vitro*.

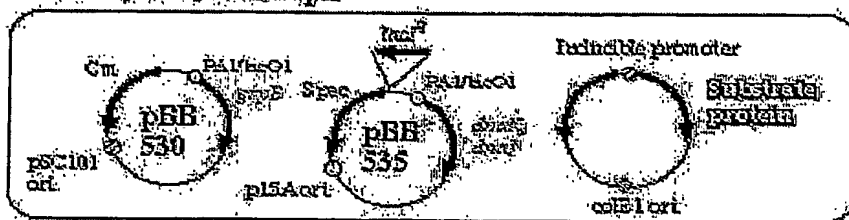
15

FIG. 1(A)

Chaperone combinations

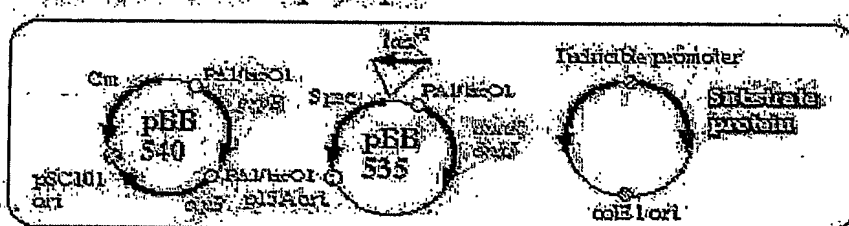
1

DnaK, DnaJ and GrpE



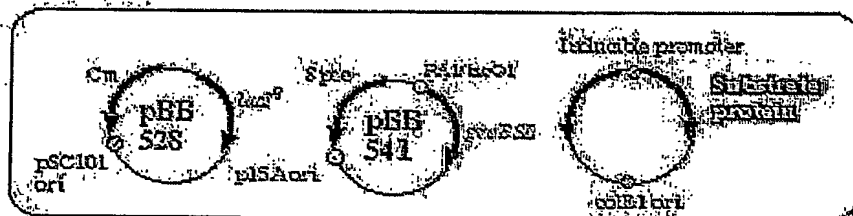
2

DnaK, DnaJ and GrpE, ClpB



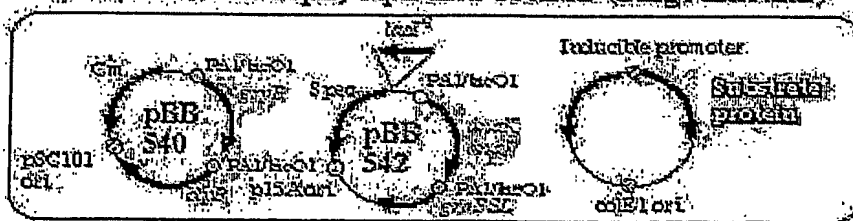
3

GroESL



4

DnaK, DnaJ and GrpE, ClpB and GroESL (Large amounts)



5

DnaK, DnaJ and GrpE, ClpB and GroESL (Small amounts)

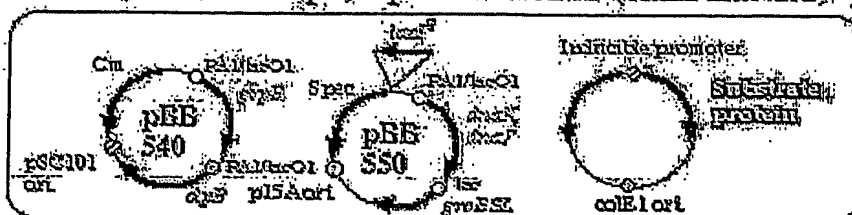


FIG. 1(B)

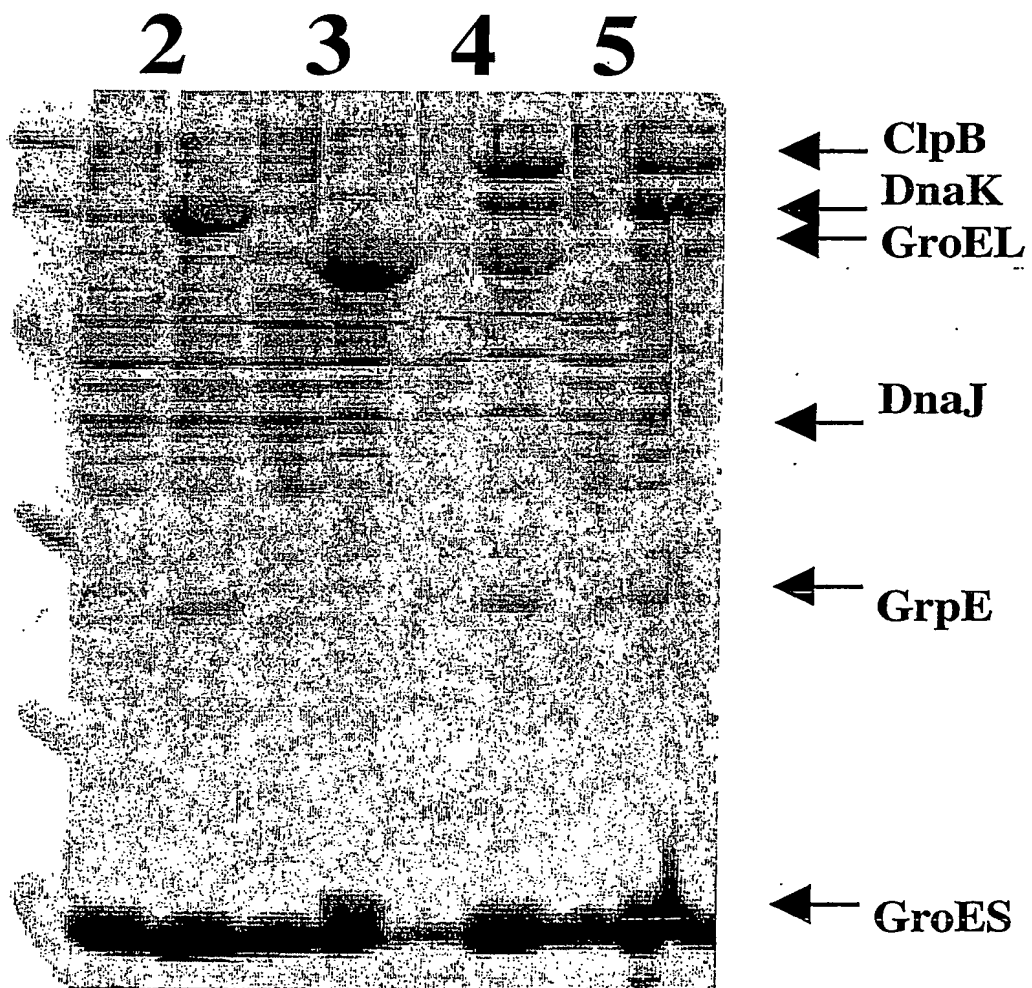


FIG. 2

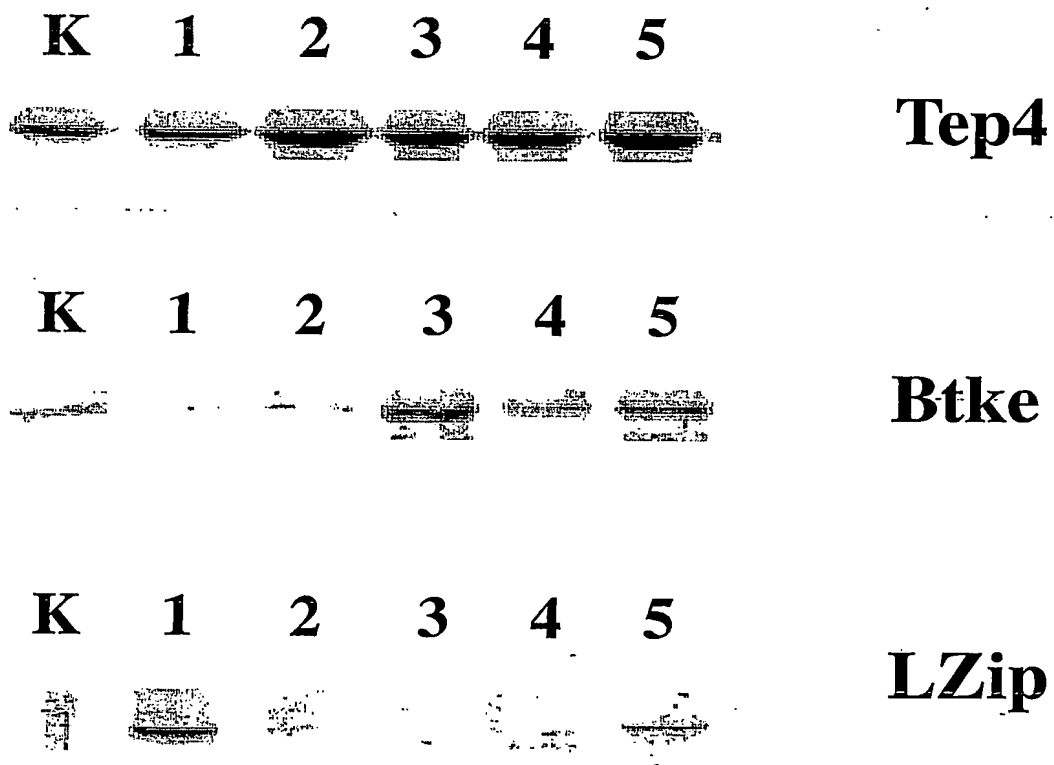


FIG. 3

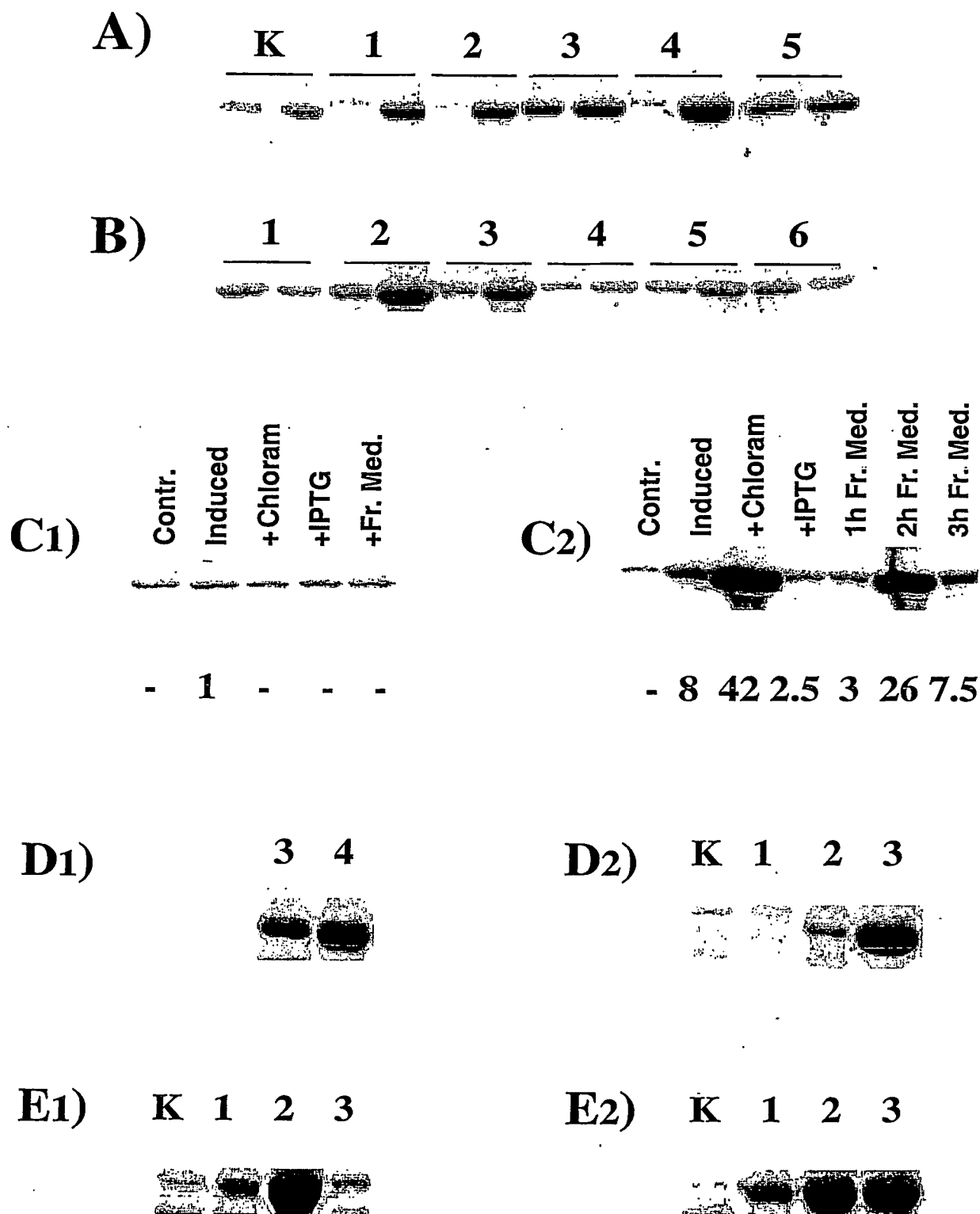


FIG. 4(A)

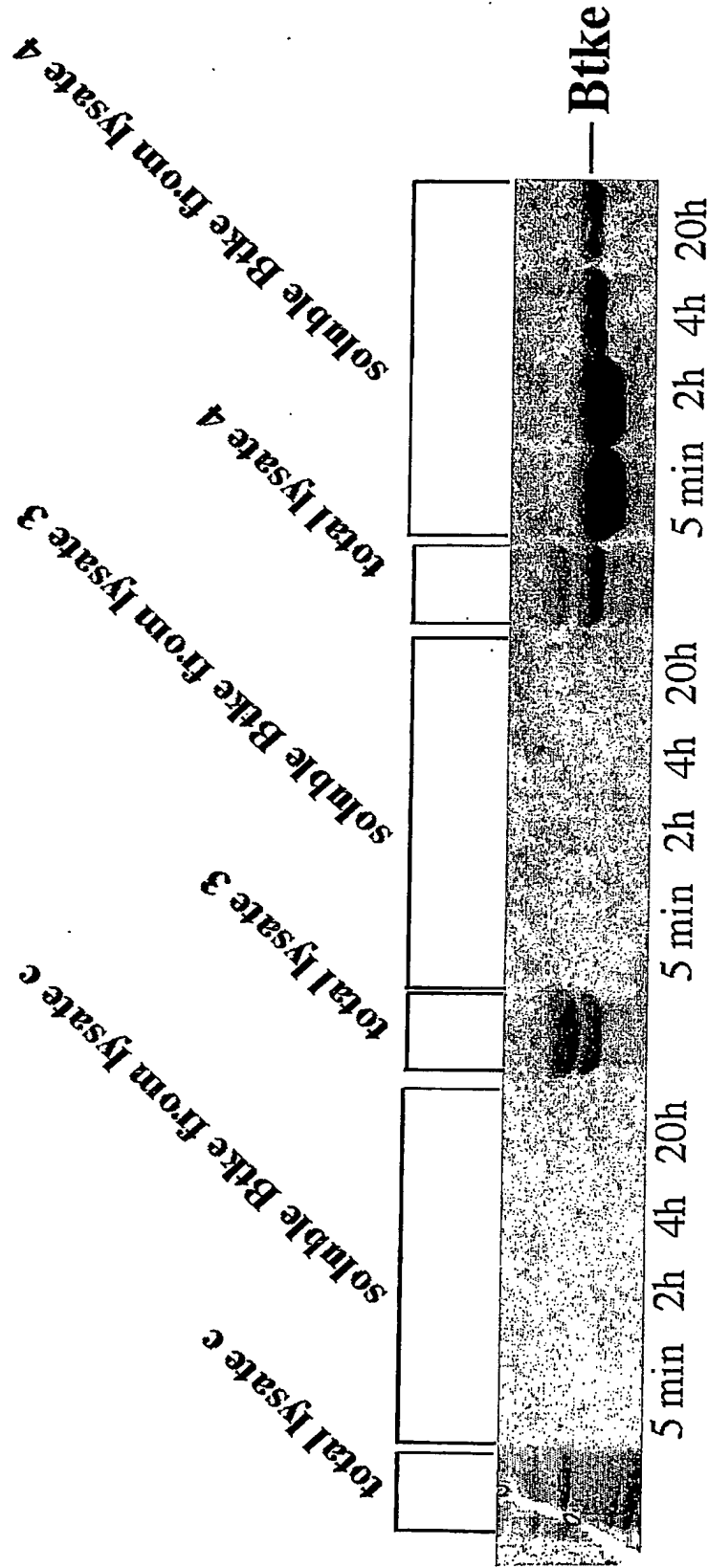
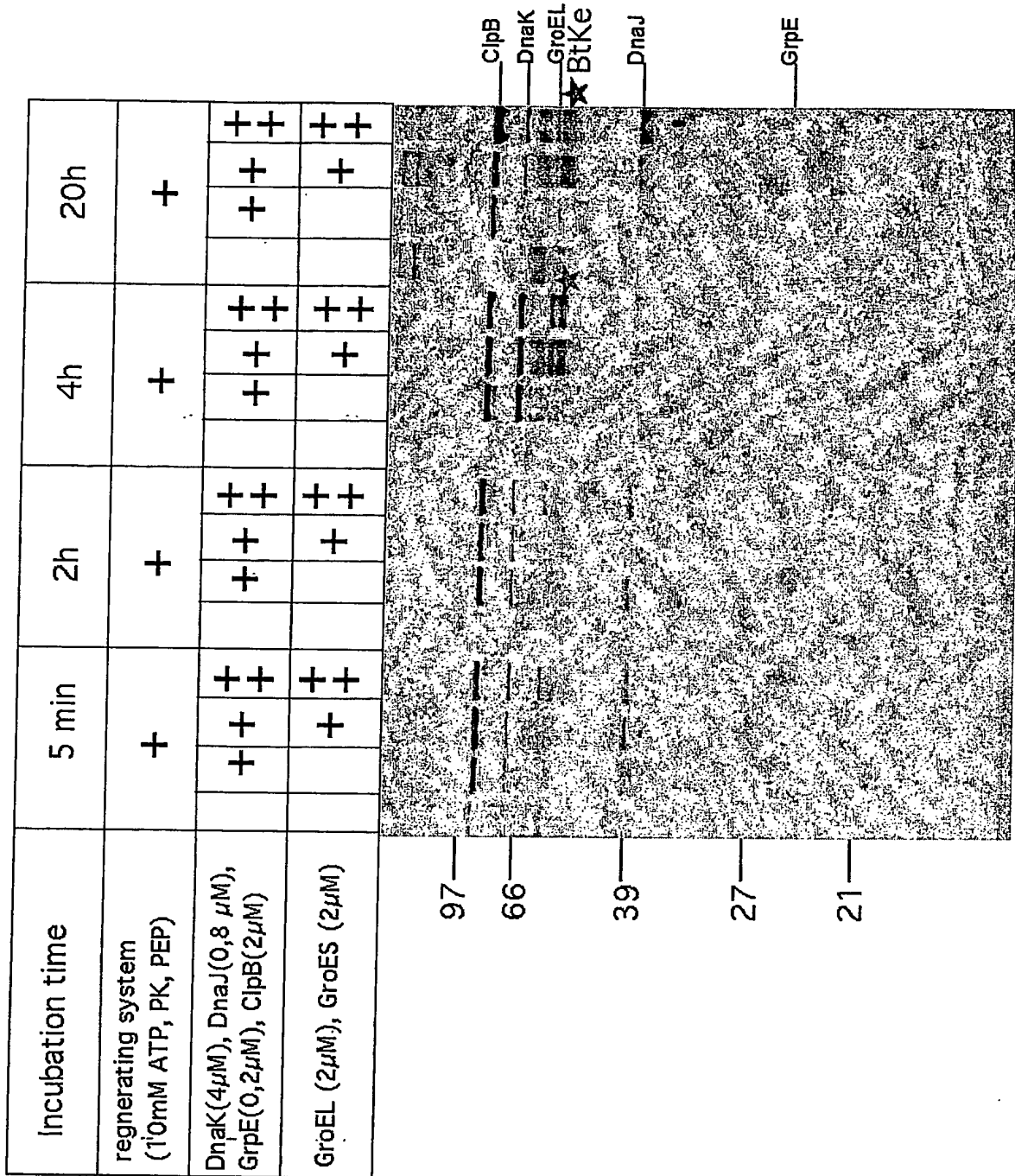


FIG. 4(B)



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